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December 11, 1996

PATENT APPLICATION  
Docket No.: UMMC91-03A2



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Harriet L. Robinson, Ellen F. Fynan, Robert G. Webster and Shan Lu  
Serial No.: 08/187,879 Group: 1804  
Filed: January 27, 1994 Examiner: C. Hogue  
For: IMMUNIZATION BY INOCULATION OF DNA  
TRANSCRIPTION UNIT

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CERTIFICATE OF MAILING	
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on <u>12/11/96</u>	<u>Martha Nelson</u>
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TRANSMITTAL OF EXECUTED SECOND DECLARATION UNDER  
37 C.F.R. 1.132 OF DR. HARRIET L. ROBINSON

Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

Transmitted herewith is an executed Second Declaration under 37 C.F.R. §1.132 of Dr. Harriet L. Robinson. The unexecuted Declaration was filed under Certificate of Mailing dated December 6, 1996.

Respectfully submitted,

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Lexington, MA 02173  
Date: December 11, 1996

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PATENT APPLICATION  
Docket No. UMMC91-03A2

Att # 20



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Harriet L. Robinson, Ellen F. Fynan, Robert G. Webster and Shan Lu

Serial No.: 08/187,879 Group Art Unit: 1804

Filed: January 27, 1994 Examiner: C. Hogue

Title: IMMUNIZATION BY INOCULATION OF DNA  
TRANSCRIPTION UNIT

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I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Assistant Commissioner for Patents, Washington, D.C. 20231

on 12/11/96 Martha Nelson

Date Martha Nelson Signature

Elizabeth M. Mahoney

Typed or printed name of person signing certificate

SECOND DECLARATION UNDER 37 C.F.R. 1.132  
OF DR. HARRIET L. ROBINSON

The Assistant Commissioner  
for Patents  
Washington, DC 20231

Sir:

I, Harriet L. Robinson, of 3 Birchwood Avenue,  
Southboro, Massachusetts 01772, hereby declare and state  
that:

1. I am a co-inventor on the above-identified patent application.

2. I was involved in the planning and performance of the following experiments, directed towards protection against measles virus by vaccination using DNA transcription units.
3. DNA Constructs for Immunization Against Measles Virus:  
A series of DNA transcription units were prepared for immunizations against measles virus. The series uses the JW4303 vectors developed at James I. Mullins laboratory (Stanford University) (Palo Alto, CA). The JW4303 vectors and accompanying oligonucleotides are designed to facilitate the cloning of PCR amplified fragments of an antigen of any isolate of measles virus. The JW4303 plasmid uses approximately 2000 bp from the cytomegalovirus (CMV) immediate early promoter and sequences from the bovine growth hormone for insert expression. Sequences from the CMV immediate early promoter include sequences encoding the CMV intron A. This intron can enhance the expression of inserted genes (Chapman, et al., Nucleic Acids Research 14:3979-3986 (1991)). The tissue plasminogen activator (TPA) leader facilitates synthesis and secretion of glycosylated proteins (Haigwood, et al., Prot. Eng. 2:611-620 (1989)). This synthetic leader provides the start site for antigen expression. Polymerase chain reaction (PCR) amplification from designer oligonucleotides is used to create antigen fragments that are inserted in-frame with the TPA leader.

Three types of antisense oligonucleotides allow construction of normal form hemagglutinin (H), a secreted form of hemagglutinin (sH), or a normal form of fusion protein. PCR was used to clone measles antigens into the vectors. The antisense oligonucleotides have unique restriction sites that facilitate cloning into JW4303 or derivatives of JW4303. Full length

hemagglutinin was cloned into HinDIII and NheI digested JW4303. Secreted hemagglutinin was inserted with a sequence for tissue plasminogen activator (TPA) into NheI digestion sites JW4303. The fusion protein construct was cloned into the vector using HinDIII and BamHI digestion sites.

4. Antibody Responses to Inoculation of Mice using H DNA, sH DNA and F DNA: The ability of hemagglutinin (both membrane-associated and secreted) and fusion glycoproteins to raise neutralizing antibody was examined. Four groups of BALB/c mice were inoculated with JW4303 plasmids: one group receiving H DNA, one group receiving sH DNA, one group receiving F DNA, and a control group receiving the plasmids with no antigen DNA insert.

To deliver the DNA, the Accell particle bombardment device (Agracetus, Middleton, WI) was employed to deliver DNA-coated gold beads to the epidermis of the mice. For gene-gun delivery of DNA to mice, plasmid DNA was affixed to gold particles by adding gold powder (Degussa, South Plainfield, NJ), and plasmid DNA to a centrifuge tube containing spermidine. Plasmid DNA and gold were coprecipitated during vortex mixing, after which the precipitate was allowed to settle and was washed with absolute ethanol and resuspended in ethanol. The gold/DNA suspension was transferred to a capped vial and immersed in a sonicating water bath to resolve clumps. Then the DNA/gold suspension was layered onto Mylar sheets and allowed to settle for several minutes, after which the meniscus was broken and excess ethanol was removed by aspiration. DNA/gold-coated mylar sheets were dried and stored under vacuum. The total amount of DNA per sheet was a function of the DNA/gold ratio. The

DNA/gold ratio was 2.5  $\mu$ g DNA/1.0 mg gold, resulting in 1  $\mu$ g DNA per sheet and 0.4 mg gold per sheet.

The BALB/c mice were anesthetized with Ketaset/Rompun. Abdominal target areas were prepared using known techniques. Target areas were thoroughly rinsed with water prior to gene delivery. DNA-coated gold particles were delivered into abdominal skin with the Accell instrument, which employs an electric spark discharge as the motive force (Yang, M.S. *et al.*, *Proc. Natl. Acad. Sci. USA* **87**: 9568-9572 (1990)). Each mouse received four deliveries per inoculation, at a discharge voltage of 17kV. The beads deliver DNA into cells, where the DNA dissolves and can be expressed. (Yang, M.S. *et al.*, *Proc. Natl. Acad. Sci. USA* **87**: 9568-9572 (1990); Williams, R.S. *et al.*, *Proc. Natl. Acad. Sci. USA* **88**: 2726-2730 (1991)). One group of six mice was inoculated with H DNA, another group of six mice was inoculated with sH DNA, another group of six mice was inoculated with F DNA, and the last group of six mice was inoculated with beads that contained control plasmids. The mice in each group were inoculated twice, with the second inoculation occurring approximately four weeks after the first.

To perform antibody assays, sera were collected immediately prior to each DNA inoculation, and at eleven times after the second inoculation. Anesthetized mice were bled from the eye vein into microhematocrit tubes.

At weeks 16 and 50, pooled sera from each test group of mice were tested for the presence of neutralizing antibodies which inhibit replication of the Edmonston strain of measles virus. These tests were conducted by incubating infectious units of Edmonston strain measles virus with various dilutions of mouse sera. After incubation, Vero cells were added. Later, the cells were washed and fed with fresh medium.

Subsequently, cultures were scored for measles virus plaques in a plaque reduction assay. The results of the assays are summarized in Table 1, below. The neutralizing titers were defined as the reciprocals of the last dilution of sera giving  $\geq 90\%$  reduction in the number of plaques.

Levels of antibodies were scored using an ELISA using known methods. See Ausubel *et al.*, Current Protocols in Molecular Biology, 2nd, John Wiley & Sons, Inc. (1995). Wells of a microtiter plate were coated with purified DOC-extracts of L-cells expressing H or F (the L-cells were provided by Dr. Fabian Wild). Dilutions of test sera were incubated in the wells and the amount of IgG scored. Appropriate substrates were added and color development evaluated using an ELISA reader to determine optical densities. Optical densities for sera of the control group (mice receiving no antigen DNA) were subtracted from values obtained for the experimental groups (mice receiving H DNA, sH DNA or F DNA).

To determine the quality of the neutralizing antibodies raised by the antigen DNA, the neutralization titer was divided by the number of ELISA units, IgG. The results are summarized in Table 1. Both DNA-expressed H and sH raised long-lived neutralizing responses, with significant levels of antibodies that persisted at fifty weeks. Furthermore, they raised neutralizing antibodies of a similar quality, as shown in Table 1.

Table 1: Quality of Neutralizing Antibody Raised by Full-Length and Secreted Forms of Hemagglutinin

DNA	Week	Neutralization titer, 90%*	ELISA units, IgG	Neut/ELISA
pJW4303/H	16	460	711	0.7
	50	340	674	0.5
pJW4303/sH	16	300	242	1.2
	50	330	287	1.1

\*Edmonston strain

To demonstrate the temporal immunological effects of the inoculations, the sera of each of the H DNA-immunized mice and the sH DNA-immunized mice were tested over a course of twenty-eight weeks for presence of neutralizing antibodies. The titrations of antibodies were done as described in Current Protocols in Molecular Biology, supra. Titors of neutralizing activity are the reciprocals of the highest dilution of sera giving complete neutralization of 200 50% tissue culture infectious doses of virus. The sera of mice from both groups demonstrated peak levels of titer four to eight weeks after the second inoculation. The sera of mice inoculated with H DNA demonstrated a high level of antibodies over the course of the twenty-eight week assay.

5. Antibody Responses to Inoculation of Mice using F DNA and H DNA: To test a vaccine which included both H and F DNA, ten test groups of BALB/c mice (six mice per group) received gene deliveries of either H DNA, F DNA, or both H and F DNA. For each type of DNA delivered (i.e., H, F, or both), one group of mice was inoculated once, and one group of mice was inoculated twice, with the second inoculation occurring four weeks after the

first inoculation. Each mouse was bled prior to each inoculation and seven weeks after the first inoculation.

The Accell particle bombardment device (Agracetus, Middleton, WI) was used to deliver DNA coated beads to the abdominal epidermis of the mice. The DNA beads were prepared as described in the previous example, but with a DNA/gold ratio of 1.0  $\mu$ g DNA to 1.0 mg gold. The mice were anesthetized with Ketaset/Rompun and the abdominal target areas were prepared using known techniques. The DNA beads were delivered to the target areas in four deliveries per inoculation, at a pressure of 450 p.s.i., resulting in each target area receiving 0.5  $\mu$ g DNA and 0.5 mg gold per inoculation.

The first two experimental groups of mice received beads coated with H DNA, group 1 receiving one inoculation, and group 2 receiving two inoculations. The next two groups received beads coated with F DNA, group 3 receiving one inoculation and group 4 receiving two inoculations. Each of the next two groups received beads coated with H DNA and beads coated with F DNA, group 5 receiving one inoculation of the separate H-coated beads and F-coated beads and group 6 receiving two inoculations of the separate H-coated beads and F-coated beads. The next two groups received inoculations where each bead was coated with a mixture of both H and F DNA, group 7 receiving one inoculation of the mixture beads and group 8 receiving two inoculations. The final two groups were controls, wherein the mice were inoculated with beads coated with pJW4303 plasmids containing no antigen inserts, group 9 receiving one inoculation of control beads and group 10 receiving two inoculations of control beads.

To determine the ability of these inoculations to raise neutralizing antibodies, the mice were bled at six weeks and the sera from the mice in each test group was

pooled and assayed for neutralizing antibodies. The neutralization titers are the reciprocals of the highest dilution of sera giving complete neutralization of 200 50% tissue culture infectious doses of virus. The sera of mice from the control group did not demonstrate appreciable neutralization titers. The other groups demonstrated considerable neutralization titers, particularly in the groups that received a second inoculation four weeks after the first inoculation. The neutralization titer for mice receiving inoculation of only beads coated with H DNA was higher than the levels raised in mice receiving beads coated with both H DNA and F DNA, whether they received the combination on the same bead or on different beads.

6. Antibody Response to Inoculation of Rabbits with H DNA and F DNA: A further experiment was undertaken in NZW rabbits using the Accell gene gun to deliver DNA coated gold beads into their abdominal epidermis.

Four rabbits were anesthetized with Ketaset/Rompun, and their abdominal target areas were prepared. Two rabbits were inoculated with pJW4303/H DNA and two with pJW4303/F DNA, using the gene gun method described 3 above, with each rabbit receiving 36 deliveries per inoculation, at a pressure of 450 p.s.i. The DNA/gold ratio was 0.5  $\mu$ g DNA/1.0 mg gold. Each target area received 0.5 mg gold and 0.25  $\mu$ g DNA per inoculation. Each rabbit was inoculated three times, at intervals of four weeks. The rabbits were bled prior to each inoculation and at varying intervals after the inoculations.

The rabbits were then analyzed for antibody responses to the H DNA administrations. Levels of antibodies were scored using ELISA. To test the serum

of DNA-immunized rabbits, the wells of a microtiter plate were coated with purified DOC-extracts of L-cells expressing H DNA (the L-cells were provided by Dr. Fabian Wild.) Dilutions of test sera were incubated in the wells and the amount of anti-H IgG was scored. Appropriate substrates were added and color development evaluated using an ELISA reader to determine optical densities. Antibodies were raised after the first inoculation and were boosted by the second inoculation.

Neutralizing activity in the H DNA-inoculated and F DNA-inoculated rabbits was also tested. The titrations of antibodies were done as described in Current Protocols in Molecular Biology, supra. Titters of neutralizing activity are the reciprocals of the highest dilution of sera giving complete neutralization of 200 50% tissue culture infectious doses of virus. The results of both the ELISA and the neutralization titer assays demonstrated that both of the rabbits immunized with H DNA presented demonstrable rises in antibodies after each inoculation, peaking within four weeks of the third inoculation. The F-immunized rabbits also demonstrated raised neutralizing antibody titers, although the titers did not rise significantly until the ninth week.

I further declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true. Moreover, these statements were made with the knowledge that willful false statements and the like made by me are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that

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such willful false statements may jeopardize the validity of  
the application or any patent issuing thereon.

Harriet L. Robinson

12/6/96

Date